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Effective Population Size Differences in *Calomys musculinus*, the Host of Junín Virus: Their Relationship with the Epidemiological History of Argentine Hemorrhagic Fever

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Abstract. Argentine hemorrhagic fever (AHF) is a serious endemic disease in Argentina, produced by Junín virus, whose host is the Sigmodontinae rodent *Calomys musculinus*. Within the endemic area, human incidence and proportion of infected rodents remains high for 5–10 years after the first appearance of the disease (epidemic [E] zone) and then gradually declines to sporadic cases (historic [H] zone). We tested the hypothesis that host populations within the E zone are large and well connected by gene flow, facilitating the transmission and maintenance of the virus, whereas those in the H and nonendemic (NE) zones are small and isolated, with the opposite effect. We estimated parameters affected by levels of gene flow and population size in 14 populations of *C. musculinus*: population effective size (N_e), genetic variability, and mean relatedness. Our hypothesis was not supported: the lowest levels of variability and of N_e and the highest genetic relatedness among individuals were found in the H zone. Populations from the NE zone displayed opposite results, whereas those in the E zone showed intermediate values. If we consider that populations are first NE, then E, and finally H, a correlative decrease in N_e was observed. Chronically infected females have a low reproductive success. We propose that this would lower N_e because each cohort would originate from a fraction of females of the previous generation, and affect other factors such as proportion of individuals that develop acute infection, probability of viral transmission, and evolution of virulence, which would explain, at least partly, the changing incidence of AHF.

INTRODUCTION

Argentine hemorrhagic fever (AHF) is a serious disease, endemic in a large part of central Argentina. The etiological agent of AHF is Junín virus—JUNV (genus Mammarenavirus, family Arenaviridae) whose host in nature is the rodent *Calomys musculinus* (Cricetidae, Sigmodontinae), one of the most abundant small mammal species in central Argentina.[1] The main transmission mechanism of JUNV among rodents is horizontal, by contact of urine, saliva, and feces from an infected animal.[2,3] Animals infected in the adult stage do not show altered survival.[4] Since the discovery of the disease, the endemic zone (defined by the occurrence of the disease in humans) has been expanding gradually. At present, it covers approximately 150,000 km^2 in four Argentinean provinces and continues to expand northward; reemergence in historical areas has also been reported recently.[5,6] The endemic zone consists of an epidemic (E) zone, characterized by the continuous presence of human cases with a mean incidence greater than 2.0/10,000 inhabitants, and a historic (H) zone, with a mean incidence of less than 2.0/10,000 inhabitants.[7–9] When the disease appears in a new area, its incidence among the human population (and the proportion of infected rodents) remains high for 5–10 years and then gradually declines to only sporadic cases.[7,10]

In zoonotic diseases, factors that influence the connections between susceptible and infected hosts are important for their transmission and maintenance.[11,12] Furthermore, if a pathogen is specialized to a single host species, it is more likely to experience frequent local extinction and recolonization events than generalist pathogens, particularly in small and fragmented wild host populations.[13] In this context, population genetics provides powerful tools to help understand zoonotic disease dynamics by providing methods to estimate population sizes and gene flow events of both pathogens and hosts.[14] For example, Guivier et al.,[15] found that Puumala virus prevalence among bank voles was positively correlated with highly connected vole populations. Besides gene flow, several authors suggested that for the horizontal transmission of JUNV among rodents to occur, a threshold population density of infected hosts and a given proportion of susceptible juveniles are necessary.[3,8,16] Polop et al.,[8,9] based on differences in rodent densities and large-scale environmental variables, hypothesized that in the E zone environmental conditions would allow the establishment of large host demes connected by high levels of gene flow. This would favor virus maintenance within populations and its transmission among them. In the nonendemic (NE) area, environmental conditions different from those in the E zone would determine small host demes connected by little gene flow, such that if the virus is introduced, there is a high chance of extinction due to low transmission rates. Piacenza[17] identified climatic and environmental differences among zones, using field and remote sensing information. The E zone had a warmer climate, with less temperature variation and more seasonality of rainfall than the H zone. Both the E and endemic zones showed more variation in the Normalized Difference Vegetation Index between seasons. The NE zone showed no differences with the E and H zones, probably related to its large extension. Piacenza[17] also found differences in the composition and diversity of species and in the relative abundance of *C. musculinus* among the three epidemiological zones. The E zone was the most diverse and the numerical dominance of *C. musculinus* over the remaining species of the rodent assemblage was clear. On the contrary, the H zone was the less

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diverse and another sigmodontine species predominated, *Akodon azarae*. In the NE zone, the species assemblage and *C. musculinus* abundance were variable, depending on the year. The author proposed that the relative abundance of the different rodent species of the assemblage would be influencing the dynamics of the pathogen. The infection in rodents of the E zone would be maintained by an increased frequency of contacts because of the greater abundance of the host with respect to other species of the assemblage. In the H and NE zones, the lower abundance of *C. musculinus* would impair the transmission of the virus by reducing the rate of encounters between individuals.

In this work, we aim to contribute to the understanding of the dynamics of AHF by using population genetics tools to investigate the proposals exposed previously. If *C. musculinus* populations within the E zone of AHF are indeed large and well connected by gene flow, they should be characterized by large effective population sizes, high levels of genetic variability, and low levels of mean relatedness as the result of the admixture among nearby demes. By contrast, populations in the NE zone should present small effective sizes, low levels of genetic variability, and high levels of relatedness between individuals as the result of small, isolated demes. We also hypothesize that H populations, which were E but currently show a very low proportion of rodent infection and human incidence of the disease, will be more similar to NE than to E populations.

MATERIALS AND METHODS

Rodents were captured using Sherman-like live traps in 14 geographic locations (Figure 1, Table 1) within NE, H and E zones at the time of sampling.[9,17] Within each of these sampling locations (from now on called "populations" for simplicity), three lines were set in each of three different crop borders separated by 2–5 km. Each trapline consisted of 20 live-capture traps, placed at 5-m intervals and baited with a mixture of peanut butter and cow fat. Samplings were performed for three consecutive nights during autumn of years 2007 and 2008, and traps were checked each morning; procedures followed biosecurity standards.[18,19] All traps containing animals were translated to a field laboratory, where rodents were anaesthetized by inhalation of methoxyflurane, and species, gender, reproductive state, and body measures were registered for each individual. A small piece of the tail tip of each *C. musculinus* individual was cut and preserved in 90% ethanol for DNA extraction. *Calomys musculinus* individuals were euthanized by methoxyflurane overdose to obtain blood and brain samples for epidemiological surveillance; other species were released back to the trapping site. Research on live animals was performed in a humane manner and followed guidelines for the care and use of animals approved by the American Society of Mammalogists.[20] Handling of rodents followed standardized safety guidelines recommended by the U. S. Centers for Disease Control and Prevention.[19]

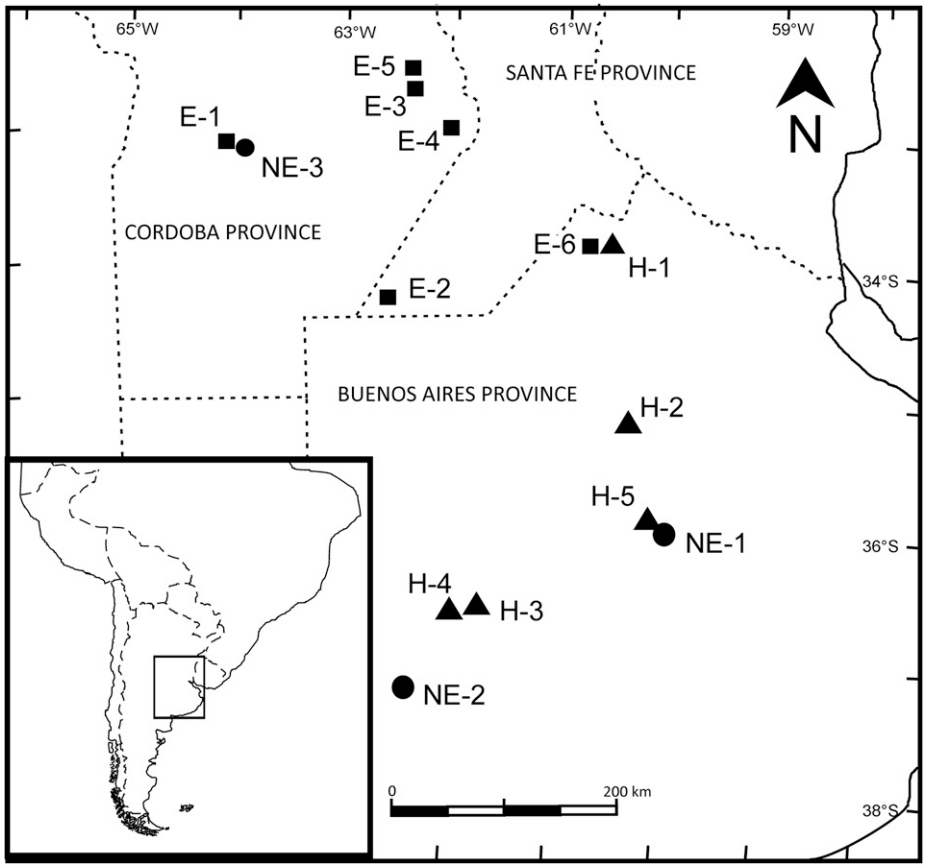

FIGURE 1. Geographic location of *Calomys musculinus* populations analyzed. Populations from nonendemic (NE) zones of Argentine hemorrhagic fever are indicated with circles, those from historic zones (H) with triangles, and those from epidemic zones (E) with squares.

TABLE 1
Sampling sites of *Calomys musculinus*

Zone	Sampling location	Geographic location	Sample size
NE	NE-1	36°0′S 60°2′W	12
	NE-2	37°11′S 62°29′W	12
	NE-3	33°3′S 63°54′W	21
E	E-1	32°59′S 64°4′W	24
	E-2	34°14′S 62°35′W	17
	E-3	32°37′S 62°20′W	28
	E-4	32°56′S 62°1′W	20
	E-5	32°28′S 62°19′W	16
	E-6	33°51′S 60°41′W	27
H	H-1	33°51′S 60°35′W	30
	H-2	35°11′S 60°21′W	20
	H-3	36°35′S 61°48′W	12
	H-4	36°37′S 61°57′W	15
	H-5	35°56′S 60°7′W	18

E = epidemic zones; H = historic zones; NE = non endemic zones.

Genomic DNA was extracted from tail tips using a salt-precipitation method.[21] A total of 211 rodents were genotyped for six microsatellite loci following Chiappero et al.[22]; polymerase chain reactions followed the protocol described therein but using fluorescently labeled forward primers. The molecular size of the amplification products was determined using an ABI3100 sequencer at Macrogen Inc (Seoul, Korea). Fragments were scored using the software PeakScanner version 2.0 (Applied Biosystems, Foster City, CA) and binned using MsatAllele,[23] which defines the bin limits based on the distribution properties of the observed fragment sizes.

Rodent populations were checked for the presence of scoring errors due to stuttering or to large allele dropouts using the software MICROCHECKER.[24] Levels of genetic variability in each population were estimated as mean expected heterozygosity (H_e) and mean allelic richness (AR). Queller and Goodnight's[25] relatedness coefficient ($r_{Q\&G}$) was calculated for pairs of individuals and averaged by population. Differences in H_e, AR, and $r_{Q\&G}$ among epidemiological zones were evaluated using the test for comparison among groups of populations implemented in FSTAT.[26] For each statistic, this test calculates the weighted average for each group of populations (i.e., for the E, H and NE populations) and then computes the difference between groups. To obtain statistical significances, 10,000 permutations allocating each sample at random to the different groups were performed, and the tested statistic was calculated from the permuted data sets. The one-sided P value is the proportion of randomized data sets giving a larger value for the tested statistic than the observed one.[26]

Current effective population sizes (N_e) were approximated by calculating Theta ($\theta = 4 \times N_e \times \mu$) for each population, using the Bayesian method implemented in the R package VAREFF.[27] First, the function "Theta" was used to obtain an estimate of the order of magnitude of θ (called θ_1) for each population. The function VarEff was then used to estimate θ for each population. Prior values for effective size for each population were set to $\theta_1/4\mu$, prior values for time was set to 30,000 generations (chosen after a series of preliminary runs), and variance for time and effective size were set to 0.5. The number of population size changes was set to 2, and we used a stepwise mutation model for microsatellites with $\mu = 5 \times 10^{-4}$.[28,29] The function was run for each population with 10,000 batches, a length of batch of 15, a space of batch of 30, and an acceptance rate of 0.25, after burning of 10,000 steps. The harmonic mean of the posterior distribution of current θ values and their 95% confidence intervals were registered for each population. Average and standard errors of θ were calculated by zone, and significance of differences between means were calculated with a one-sided t test using Infostat.[30] In addition, to rule out a possible correlation of the estimated θ's with sample sizes (N), we tested the correlation between θ and N with the Spearman test using Infostat.

RESULTS

Microchecker analysis detected no scoring errors due to stuttering or large allele dropouts. Mean levels of genetic variability in each epidemiological zone are presented in Table 2. Allelic richness was, on average, significantly higher in the NE than in the H or E zones ($P = 0.003$ and $P = 0.023$, respectively), whereas H_e was significantly lower in the H zone compared with the NE and the E zones ($P = 0.032$ and $P = 0.035$, respectively). Individuals within the H zone were on average more related than individuals within the NE zone ($P = 0.016$).

Lower population effective sizes were observed in central and northern Buenos Aires Province (Figure 2A). Most of these populations are within the H zone of AHF, except NE-1, and E-6 that are very close to H localities (H-5 and H-1, respectively). Mean θ was significantly higher in the NE and E zones than in the H zone (Figure 2B). No correlation was found between sample size and θ (Spearman correlation coefficient = 0.042; $P = 0.887$).

DISCUSSION

The analysis of genetic structure of host populations can be of great importance to understand the spread of a zoonosis, especially for pathogens such as viruses that can survive outside the host for a very short time. In this case, contacts between infected and noninfected animals would be the only way for the pathogen to persist within and to spread among populations. It has been long established that when AHF appears in a new area, both its incidence among the human population and the proportion of infected rodents remains high for 5–10 years (E populations) and then gradually declines to only sporadic cases (H populations).[7] In this study, we worked on the hypothesis that populations within the E zone of AHF, would be large and well connected by gene flow, favoring the persistence and spread of JUNV. On the contrary, those in the H and NE zone would be smaller and more isolated, dampening the maintenance of the virus. Therefore, we expected populations within these zones to show lower levels of effective population size (N_e) and genetic variability and higher mean relatedness compared with populations within the E zone. However, our results were not fully compatible with these expectations: populations from the H zone of AHF

TABLE 2
Average levels of genetic variability and relatedness by epidemiologic zone in *Calomys musculinus*

	AR			H_e			$r_{Q\&G}$		
Nonendemic	8.547	A	–	0.834	A	–	0.005	A	–
Epidemic	8.014	–	B	0.835	A	–	0.026	A	B
Historic	7.899	–	B	0.814	–	B	0.034	–	B

AR = allelic richness; H_e = mean expected heterozygosity; $r_{Q\&G}$ = Queller and Goodnight's (1999) relatedness coefficient. Different letters indicate significant differences at $\alpha = 0.05$ using the test for comparisons among groups of samples in FSTAT.

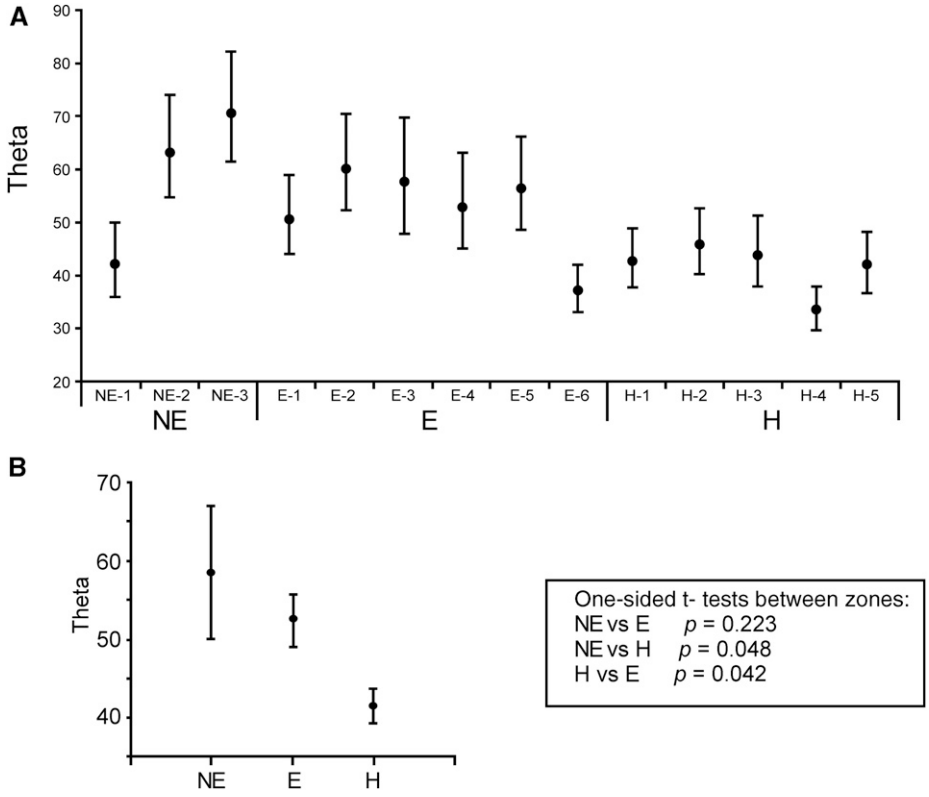

FIGURE 2. (**A**) Current θ ($4N_e\mu$) values obtained with the program Vareff[27] for 14 populations of *Calomys musculinus*. Circles indicate the harmonic mean of the posterior distribution of theta current times and vertical bars, the 95% confidence interval. (**B**) Average θ by epidemiological zone ± SE. E = epidemic zones; H = historic zones; NE = nonendemic zones.

showed indeed the lowest levels of genetic variability (measured as H and AR) and of N_e, and individuals were on average highly related to each other but compared with populations in the NE zone, which showed the highest H, AR, and N_e and low levels of mean relatedness. Epidemic populations presented high levels of H and N_e but low AR, and mean relatedness was intermediate between that of the NE and the H zones. In other words, if we consider that the progression of a given population flows from NE to E to H, a simultaneous increment of the consequences of decreasing effective population size can be observed.

Several explanations have been considered to account for the geographic expansion of the endemic area and the changing incidence of AHF: changes in human agricultural practices that resulted in the creation of ideal conditions for the expansion of opportunistic species,[31] patterns of migration of *C. musculinus*,[32,33] and changes in the virulence of JUNV strains.[34] Piacenza[17] found that *C. musculinus* was numerically dominant over the remaining species of the rodent assemblage in E populations, but in H ones the dominant rodent species was *A. azarae*. In NE populations of central Buenos Aires Province (NE-1 and NE-2, Figure 1) *A. azarae* was the dominant species, whereas in NE-3 (Córdoba Province, Figure 1) it was *C. musculinus*.[17] The author proposed that climatic and environmental differences among zones would imply differences in available habitats for the species and in its quality, which would translate into differences in abundance. They further suggested that the infection among rodents in the E zone would be maintained by an increased frequency of contacts because of the greater abundance of the host. In the H (and NE) zone, the low abundance of *C. musculinus* would reduce the frequency of intraspecific contacts and the possibility of virus transmission among rodents, according to the dilution effect.[35] Our results indicate that the lower abundance of *C. musculinus* in the H compared with the E zone is correlated with lower effective population sizes, with the genetic consequences of low variability and higher relatedness between individuals, supporting the hypothesis of smaller, more isolated populations in the H zone. Our hypothesis proposed a similar scenario for NE populations, but the only one that supported it was NE-1, which is geographically very close to H population H-5 (Figure 1). Contrary to our hypothesis, the other two populations in the NE zone (NE-2 and NE-3) have the highest effective sizes and levels of variability and the lowest relatedness between individuals of the three zones (Figure 2, Table 2).

There is another perspective that has to be explored to explain the smaller effective sizes of *C. musculinus* populations in H, compared with E and NE areas. When infected with JUNV, adult *C. musculinus* experience an acute phase characterized by active virus replication. Thereafter, some individuals clear the infection whereas others shift to a lifelong chronic phase. These rodents continuously shed the virus into the environment through body fluids, being the primary reason for the maintenance of JUNV in nature.[36] They are asymptomatic and do not show differences in cumulative mortality, body weight gain or reproductive patterns compared with controls.[3,37] However, other virus/rodent systems with apparently asymptomatic infections suggest that consequences of a viral infection can be subtle. A decreased immune response when

exposed to cold conditions, less overwinter survival,[38,39] and differences in reproductive success[40–42] of infected rodents compared with controls were reported in hantaviruses host species. Unfortunately, studies on the consequences of infection in free living populations of *C. musculinus* are lacking but because this species experiences significant drops in population numbers during winter[34,43] a differential overwinter survival cannot be ruled out. In *C. musculinus*, Vitullo and Merani[37] observed that JUNV does not infect embryos during gestation and neither is transferred to newborns during birth but that approximately 50% of pups nursed by mothers with chronic infection acquired the virus during lactation. Vitullo et al.,[4] found that newborns inoculated by nasal instillation 24–48 hours after birth showed lower weight gain during lactation, increased mortality rate after weaning and decreased fertility compared with controls. These results suggest that, even if survival and reproduction is not impaired by JUNV when acquired in the adult stage, females with chronic infection would have a decreased reproductive success and would contribute to the next generation in a lower proportion, than those that experience an acute infection and clear the virus.[4,37,44] If the capability to develop chronic or acute infection were genetically determined, this differential fitness would cause that 1) each cohort would be born from a fraction of the females of the previous generation, decreasing the effective population size and 2) the population will be composed by increasingly more individuals that experience an acute infection, decreasing the probability of transmission of JUNV among rodents. If this is combined with environmental characteristics that determine lower abundances of the host, the infection will slowly clear from the population.

On the other hand, Calderón[34] compared the rate and type of infections (persistent versus acute) in rodents from several endemic localities during the decade of 1990 with those of previous decades. She found that viral strains became less virulent, rodents showed a decreasing infection rate, and that in more recent samples, most infections were acute, with a short period of virus excretion. She proposed that the changing abundance of the host reduced the possibility of transmission of the virus among rodents, which would contribute to the selection of less virulent strains that do not produce persistent infections. Our results could be explained by differential reproductive success between noninfected and chronically infected females, which would be another factor determining a decrease in the proportion of individuals capable to develop chronic infection, and therefore would also contribute to select less virulent strains in nature.

In this study, we provide a rationale hypothesis to explain the dynamics of the expansion and changing incidence of a viral disease, the AHF, on the basis of field population genetic studies in the rodent acting as host. Further studies on variation of major histocompatibility complex genes in natural populations of the host and on the heritability of the immune response of *C. musculinus* to different types of infection with JunínJUNV would greatly contribute to clarify host-pathogen interactions in the natural system.

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